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Immobilization of dermatan sulphate on a silica matrix and its possible use as an affinity chromatography support for heparin cofactor II purification

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ABSTRACT

Dermatan sulphate (DS) is a glycosaminoglycan which catalyses specifically thrombin inhibition by a plasmatic inhibitor, Heparin cofactor II (HCII). DS was insolubilized on a silica matrix to study its interaction with HCII. The immobilization of DS was performed with a good yield on a silica previously coated with polysaccharides in order to neutralize the negatively charged silanol groups. The value of the affinity constant of insolubilized DS for HCII, measured by the adsorption isotherm, is consistent with the value obtained for soluble DS. The DS bound to the silica matrix was also tested as a chromatographic support for the purification of HCII from human plasma: the optimum conditions for HCII adsorption and desorption were determined. The eluted HCII was obtained with a good yield (21%) and with no contamination by antithrombin III, the other main plasmatic inhibitor of thrombin.

INTRODUCTION

Heparin cofactor II (HCII) is a human plasma glycoprotein, the properties of which were reported in the 1980s [1,2]. Although its physiological role is not yet well established, it is known that HCII inhibits thrombin in the presence of heparin or dermatan sulphate (DS) [2,3]. HCII has a structure and a mechanism of inhibition

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close to those of antithrombin III (ATIII), a well known potent thrombin inhibitor. However, these two plasmatic inhibitors have different properties: whereas ATIII inhibits all the serine proteases of the intrinsic coagulation cascade [4], HCII activity is specific to thrombin. The inhibition by HCII is specifically catalysed by DS, a glycosaminoglycan (GAG) of the vascular endothelium, which has no effect on thrombin inhibition by ATIII.

The potentiation of HCII by DS is related to a specific polysaccharide sequence [5,6], as a minimum of twelve residues is required for the HCII catalysis whereas an octasaccharide is sufficient to bind to HCII. The presence of sulphate groups in the DS structure appears to be of importance in HCII catalysis [7]. One Lys and four Arg residues are involved in the GAG binding site of HCII [8,9]. However, the precise mechanism oof interaction between HCII and DS is not yet known. The low plasma concentration of HCII and its chemical properties close to those of ATIII do not allow an easy purification process. Most of the procedures used to isolate HCII [2,9,10] involve several steps (at least five) including a first step of protein fractionation followed by ion-exchange and gel permeation chromatography; HCII and ATIII are separated by their different affinities for heparin on an insolubilized heparin sorbent. The contaminating ATIII can also be eliminated on an anti-ATIII immunoabsorbent.

In this work, we studied the interaction between insolubilized DS and HCII in order to understand better the specificity and binding mechanism between this GAG and HCII. DS was insolubilized on a dextran- or agarose-coated silica support which combines all the advantages of traditional supports and the important mechanical properties of silica [11]. The affinity of the immobilized DS for HCII was determined from the adsorption isotherm under static conditions. Then, preliminaty studies of the isolation of HCII from human plasma were carried out in order to set up an easy, fast and efficient purification procedure.

EXPERIMENTAL

Materials

Silica beads XO15M, purchased from IBF Biotechnics (Villeneuve la Garenne, France), had an average pore diameter of 1250 Å and were spherical (40–100 μm). The polysaccharides used for the coating were dextrans T500 (Pharmacia France, Bois d'Arcy, France) and Indubiose agarose A37 HAA (IBF Biotechnics). The activating agents were 1,4-butanediol diglycidyl ether (BDGE) and 1,1′-carbonyldiimidazole (CDI). Dermatan sulphate NF 112 from bovine intestinal mucosa was provided by Pharmuka (Gennevilliers, France). Human purified thrombin, chromothrombin and monospecific antibodies against HCII were purchased from Diagnostica Stago (Asnieres, France); bovine factor Xa from Biotrol (Diagnostic Reagents, U.K.) and chromogenic substrate, S2222 from Kabi-Vitrum (Stockholm, Sweden). A pool of platelet-poor plasma was prepared by centrifugation at 2000 *g* of blood from fifteen normal subjects, collected on 0.13 *M* sodium citrate. This pool was used to establish the adsorption isotherm and the calibration graphs for HCII and ATIII assays.

Preparation of coated silica supports and immobilization of DS

The synthesis of the active supports was realized in two successive steps. In the

first, silica beads were coated with dextran or agarose substituted with DEAE functions in order to neutralize the ion-exchange capacity of native silica, as described previously [11,12]. Two types of coated silica were prepared: the silica with a single layer of substituted polysaccharides as described above, and the same silica with a double coating in which the second layer consisted of a neutral polysaccharide [13]. Silica with a double coating is a new type of support; preliminary studies have demonstrated a decrease in the non-specific interactions with these supports. The amount of polysaccharides was determined by elemental analysis of carbon. In the second step, the coated silica phases were activated and coupled with DS. Before immobilization of DS, the contaminating heparin was removed from DS by a deamination treatment with nitrous acid [14]. The coated silica supports were then activated by hydroxyl groups with either 0.3 g of CDI or 3 μl of BDGE per gram of coated silica. DS coupling was performed by using 3 g of activated support and 250 mg of GAG suspended in 14.5 ml of 0.1 *M* sodium carbonate (pH 8.7). The amount of bound DS was determined by elemental analysis of the sulphur content.

Adsorption isotherm and determination of affinity constant

Adsorption experiments were performed at 4°C to prevent the loss of HCII activity. The isotherm was established from measurement of HCII adsorption using the following procedure: 400 μl of the support suspension were incubated with 0.02 *M* Tris–HCl (pH 7.4)–0.05 *M* NaCl buffer. Several dilutions of plasma, corresponding to different HCII concentrations (0.02–0.4 U/ml), were incubated with the support suspension for 90 min at 4°C. After sedimentation, the amount of residual HCII in the supernatant was determined by an HCII assay, as described under *Chromatographic experiments*. The amount of adsorbed protein corresponds to the difference between the initial and remaining concentrations of active HCII in the supernatant. The adsorption isotherm was then established. The affinity constant was calculated from the initial slope and plateau of the isotherm and the binding capacity of the support was measured from the plateau value.

Chromatographic experiments

The chromatographic experiments were performed using an LKB system. A 13 × 1.1 cm I.D. column containing 5 ml (2.5 g dry weight) of the affinity sorbent was packed using a slurry method. Each support was washed with 25 ml of initial buffer. In order to define the optimum conditions for the interaction between HCII and its ligand, different buffers (0.02 or 0.05 *M* Tris–HCl) with pH values of 6.5, 7.4 or 8.5 and NaCl concentrations of 0 or 0.05 *M* were tested. A 5-ml aliquot of human plasma was loaded on the column. Elution was performed at a flow-rate of 20 ml/h at 4°C using a 40-ml linear gradient increasing from the molarity of the initial buffer used to 1 *M* NaCl. The eluted proteins were detected at 280 nm and the chromatographic fractions collected to perform ATIII and HCII assays. The protein concentration was measured by Lowry's method [15]. The biological activity of HCII in the eluted fractions was assayed at 37°C by measuring the thrombin inhibition in the presence of DS as follows: 150 μl of a diluted sample were added to 150 μl of 0.05 *M* Tris–HCl (pH 8.4)–0.1 *M* NaCl containing 0.2 g/l of DS. After incubation for 2 min with 150 μl of human purified thrombin (3 U/ml), 150 μl of chromothrombin (1.5 μM) were added and the amidolysis of the substrate was measured at 405 nm. The results were

reported with respect to a calibration graph established under the same conditions, with dilutions of a normal plasma. By definition, the concentration of plasmatic HCII is 1 unit/ml. The biological activity of ATIII was determined by measuring its anti-factor Xa activity in the presence of heparin: 100 μl of a diluted sample were added to 100 μl of 0.05 *M* Tris–HCl (pH 8.4)–0.05 *M* NaCl containing 6 U/ml of heparin and incubated for 2 min. Then 100 μl of bovine factor Xa were added and after incubation for 1 min 100 μl of S2222 (4 m*M*) were added. Hydrolysis of the substrate was measured at 405 nm. The calibration graph was established under the same conditions, using the same pool of normal plasma as previously described. The concentration of HCII antigen was measured by electroimmunodiffusion [16] with monospecific antibodies raised against human HCII. HCII purity was checked by immunoelectrophoresis [17] with anti-human HCII and anti-human serum antibodies and by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [18].

RESULTS AND DISCUSSION

Characterization of the affinity supports

To neutralize the negatively charged silanol groups on the silica surface, a polysaccharide (dextran or agarose) was used to coat the silica. The characteristics of the coated silica are presented in Table I. A larger amount of polymer is bound to the silica when the coating polysaccharide is dextran. However, the coverage of the silica surface is more efficient with agarose, which has a greater number of DEAE groups.

The immobilization of DS was performed using CDI or BDGE as activating agents. Table II shows the amounts of immobilized DS for three differents supports. Coupling of DS by BDGE seems more efficient than that by CDI. BGDE mainly activates hydroxyl groups whereas CDI acts essentially on primary amino groups. The greater efficiency of BDGE shows that DS is mainly immobilized by its hydroxyl groups rather than by its primary amino groups. This is certainly due to the large number of hydroxyl groups on the DS compared with the small number of primary amino groups, which are mostly acetylated amino groups.

The affinity of insolubilized DS for HCII was studied by measuring the affinity constant from the Langmuir isotherm. Fig. 1 shows the adsorption of HCII on the affinity support. The adsorption isotherm obtained corresponds to the Langmuir model. The value of the affinity constant for HCII is $7 \cdot 10^5$ l/mol, which is consistent with the published value for soluble DS of $6.6 \cdot 10^5$ l/mol according to Pratt *et al.* [19].

TABLE I

CHARACTERISTICS OF COATED SILICA SUPPORTS

Support	Coating polymer	DEAE (%)	Amount of fixed polymer (mg/g silica)
SiD-DS 1	DEAE-dextran	4	121
SiD-DS 2	DEAE-dextran	4	138
ASiA-DS 1	DEAE-agarose	10	45
	Agarose	–	35

TABLE II

INSOLUBILIZATION OF DERMATAN SULPHATE ON COATED SILICA

Support	Activating agent	Amount of dermatan used (mg)	Amount of dermatan fixed (mg)	Yield (%)
SiD-DS 1	BDGE	250	195	78
SiD-DS 2	CDI	250	133	53
ASiA-DS 1	CDI	250	94	38

these similar values indicate that the non-specific interactions make only a small contribution to the overall adsorption process. Moreover, it seems that the binding sites for HCII in the DS structure are not masked or are not involved in the immobilization of DS on the coated silica. Compared with the affinity constant of soluble DS for ATIII, which is $1 \cdot 10^3$ l/mol [19], the affinity constant of HCII is higher and shows that DS has a very weak affinity for ATIII. However, the affinity constant of insolubilized DS for HCII is not in the range of 10^7 or 10^8 l/mol, which would demonstrate a very high affinity for HCII. As reported in the literature, the native DS used in this study has a weak affinity for HCII; this could be explained, as was noted by Griffith and Marbet [20], by the fact that perhaps only a small fraction of the DS is involved in the HCII binding. The binding capacity of the tested supports was of 0.5 U of HCII per gram of support.

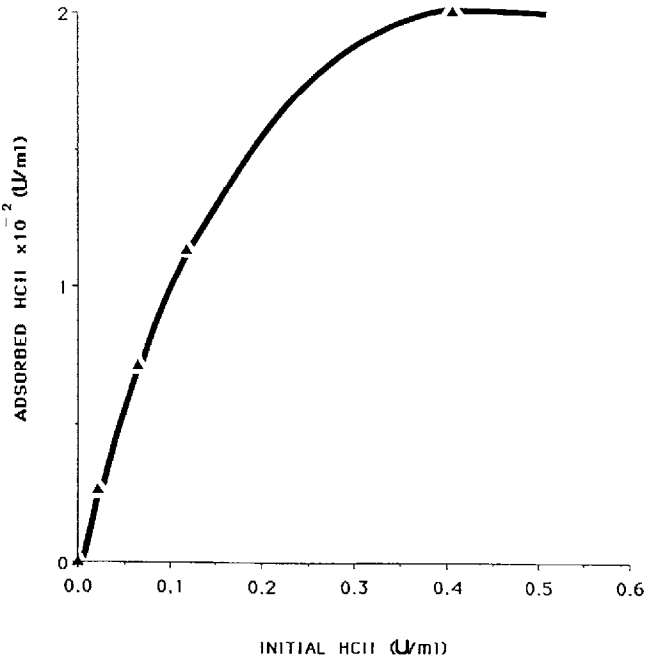

Fig. 1. Adsorption isotherm of HCII on the insolubilized DS support.

TABLE III

INFLUENCE OF INITIAL BUFFER ON THE HCII AND ATIII ADSORPTION ON AFFINITY SORBENT

Initial buffer	ATIII adsorbed (U/ml)	HCII adsorbed (U/ml)
0.02 *M* Tris (pH 7.4)	0	0.30
0.05 *M* Tris (pH 7.4)	0	0.81
0.02 *M* Tris (pH 7.4)–0.05 *M* NaCl	0	0.84
0.02 *M* Tris (pH 6.5)–0.05 *M* NaCl	0.03	0.01
0.02 *M* Tris (pH 8.5)–0.05 *M* NaCl	0	0

Isolation of HCII from human plasma

Several initial buffers were used to determine the optimum conditions of adsorption of HCII on the affinity sorbent (Table III). A minimum concentration of 0.05 *M* is required for optimum adsorption of HCII on the insolubilized DS. A buffer of 0.02 *M* [0.02 *M* Tris–HCl (pH 7.4) led to a very weak adsorption of HCII whereas buffers of 0.05 *M* Tris–HCl (pH 7.4) and 0.07 *M* [0.05 *M* Tris–HCl (pH 7.4)–0.02 *M* NaCl] allow a better adsorption of HCII. From these results and those of Sculy *et al.* [7], who showed that a maximum activity of HCII, in the presence of soluble DS, was obtained with 0.05 *M* NaCl, it seems that the optimum buffer for HCII adsorption is 0.02 *M* Tris–HCl (pH 7.4)–0.05 *M* NaCl. Under these optimum conditions, up to 21% of the initial HCII is adsorbed on the insolubilized DS. This low molarity required to obtain optimum adsorption of HCII is consistent with the weak affinity constant of the support for this inhibitor. At pH 8.5, the total activity of ATIII and HCII is recovered in the washing buffer, showing that at a basic pH neither HCII nor ATIII is adsorbed on the affinity sorbent. In contrast, when the pH is lowered to 6.5, the two inhibitors adsorbed on the insolubilized DS. The influence of pH and molarity on the adsorption of HCII on DS seems to indicate that the interaction between the inhibitor and the DS is mainly an ionic interaction. This interaction is probably due to the negative sulphate and carboxyl groups of DS, and also to the positive amino groups of HCII Lys residues.

In all the chromatographic experiments, under conditions of optimum pH and molarity, ATIII was never adsorbed on the affinity sorbent. The total amount of ATIII loaded on the column was always recovered in the washing buffer. Under these dynamic conditions, insolubilized DS has no affinity for ATIII, which is not adsorbed on the support. Thus, on our supports, we can separate HCII from ATIII. Whereas ATIII is totally recovered in the washing buffer, HCII binds to the support and can be eluted with a buffer gradient. HCII is eluted in a range from 0.12 to 0.48 *M* NaCl (Table IV) from the immobilized DS whereas is eluted from insolubilized heparin between 0.13 and 0.28 *M* NaCl. A higher molarity seems to be required to desorb HCII from insolubilized DS than from insolubilized heparin, which means that insolubilized DS has a greater affinity for HCII than for insolubilized heparin. The specific activities of the eluted HCII (mean of three experiments for each support) were 0.05

TABLE IV

CONDITIONS AND YIELD OF HCII ELUTION

Support	Molarity of HCII desorption (*M* NACl)	HCII yield (%)[a]	ATIII yield (%)
SiD-DS 1	0.12–0.38	15	0
SiD-DS 2	0.28–0.40	7	0
ASiA-DS 1	0.21–0.48	17	0

[a] Mean values of three experiments.

U/mg for SiD-DS2, 0.22 U/mg for ASiA-DS1 and 0.34 U/mg for SiD-DS1. The highest specific activity of the eluted HCII is obtained with the SiD-DS1 support; this is probably due to a lower non-specific interaction of this support than the others. However, the specific activities of the eluted HCII are not very high compared with those obtained in the classical purification processes in which specific activities of 0.9 [10] to 10 U/mg [2] were obtained. The HCII activity yields obtained with our supports were 7% for SiD-DS2, 17.5% for SiD-DS1 and 21% for ASiA-DS1, whereas no ATIII activity was found in the elution gradient. These yields are in the same range as those obtained in the previously described purification procedures of 3–30% yields [9,10]. Although SiD-DS1 and ASiA-DS1 were not prepared under the same conditions, these two supports gave comparable yields and specific activities.

The purified HCII was tested by electroimmunodiffusion. The HCII activity/HCII antigen ratio was between 0.6 and 1; a ratio of less than 1 indicates partial inactivation of the HCII after the elution with a salt gradient. The eluted HCII was also tested by SDS-PAGE and immunoelectrophoresis, which revealed two contaminating proteins, IgA and an α_2-globulin, which has not been identified as another coagulation inhibitor (ATIII or α_2-macroglobulin).

CONCLUSIONS

The interaction between DS and HCII is not well understood. It was interesting to study this interaction by immobilizing this GAG on a silica support. DS has been insolubilized on a silica phase, a synthesis which has never been achieved before. Silica beads were previously coated with polysaccharides in order to neutralize silanol groups, according to data obtained in our laboratory [13]. DS can be immobilized on silica, regardless of the coating polymer used, dextran or agarose. The GAG seems to bind to the matrix by its hydroxyl groups more than by its amino groups.

Under static conditions, the affinity constant of insolubilized DS for HCII is similar to that of soluble DS. When used as a chromatographic support under optimum conditions of pH and molarity, insolubilized DS has no affinity for ATIII. HCII is adsorbed on the affinity sorbent and can be eluted by a salt gradient between 0.12 and 0.48 *M* NaCl. With only one chromatographic step, the yield of the eluted HCII is identical with those obtained in previously described purification processes, with no contaminating ATIII. However, the specific activity is lower than those given by other published procedures.

We have shown that insolubilized DS, used as an affinity chromatography support, may allow the separation of HCII from ATIII, which is important for the study of each inhibitor. However, the native DS, soluble or insolubilized, has a weak affinity for HCII and it will be of interest to isolate a fraction of this GAG with a high affinity for HCII. The insolubilization of such a fraction might allow a very efficient isolation procedure for HCII in a single chromatographic run.

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